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TITLE: ION CHANNELS AS TARGETS TO TREAT CYSTIC FIBROSIS LUNG DISEASE

S. Lorraine Martin^{a, *}, Vinciane Saint-Criq^b, Tzyh-Chang Hwang^c and László Csanády^d.

^a School of Pharmacy, Queen's University Belfast, Northern Ireland, UK; ^b Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle upon Tyne, UK; ^c Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211, USA and ^d Department of Medical Biochemistry, Semmelweis University, Budapest 1094, Hungary.

Corresponding author:

Professor S. Lorraine Martin
School of Pharmacy
Queen's University Belfast
97 Lisburn Road
Belfast
Northern Ireland
BT44 9HB

Tel: +44 02890 975711
Email: l.martin@qub.ac.uk

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Contents

1. Introduction
 2. Recent developments in our understanding of CFTR structure and function
 3. Targeting ENaC
 4. Alternative ion channels and transporters in CF
 5. Conclusion
- Conflict of interest
Acknowledgements
References

Abstract

Lung health relies on effective mucociliary clearance and innate immune defence mechanisms. In cystic fibrosis (CF), an imbalance in ion transport due to an absence of chloride ion secretion, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) and a concomitant sodium hyperabsorption, caused by dysregulation of the epithelial sodium channel (ENaC), results in mucus stasis which predisposes the lungs to cycles of chronic infection and inflammation leading to lung function decline.

An increased understanding of CFTR structure and function has provided opportunity for the development of a number of novel modulators targeting mutant CFTR however, it is important to also consider other ion channels and transporters present in the airways as putative targets for drug development. In this review, we discuss recent advances in CFTR biology which will contribute to further drug discovery in the field. We also examine developments to inhibit the epithelial sodium channel (ENaC) and potentially activate alternative chloride channels and transporters as a multi-tracked strategy to hydrate CF airways and restore normal mucociliary clearance mechanisms in a manner independent of CFTR mutation.

Abbreviations:

ABC: ATP Binding Cassette
AE: anion exchanger
ASL: airways surface liquid
ATP: adenosine triphosphate
CAP: channel activating protease
CaCC: calcium activated chloride channel
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
ΔF508: CFTR mutation encoding a deletion of phenylalanine at position 508
ENaC: epithelial sodium channel
HAT: human airways trypsin-like protease
NBD: nucleotide binding domain
NHE: Na⁺/H⁺ exchanger
NKCC1: Na-K-Cl cotransporter
PKA: Protein kinase A
siRNA: small-interfering RNA
UTP: uridine triphosphate

1. Introduction:

Cystic fibrosis (CF) is the most common life-limiting, hereditary condition which affects Caucasian populations with morbidity and premature mortality associated predominantly with chronic lung disease [1]. It is caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene which encodes an ATP-dependent, apical membrane-associated chloride ion channel which plays a pivotal role in the regulation of ion secretion and absorption across epithelial cells. There are currently over 2000 known CFTR mutations, although fewer than 20 mutations occur at a frequency of more than 0.1% and only 5 at a frequency greater than 1% [2]. These mutations are grouped into 6 classes depending on the degree to which the CFTR mutation affects CFTR quantity, transport to or function at the cell surface, however as our understanding of CFTR structure and function increases, further sub- or re-classification may assist current aspirations for a fully personalized medicines approach to this disease [2].

The CF phenotype, which in addition to the lungs, affects the pancreas, liver, kidneys and intestines is however not just the result of abnormal CFTR-mediated Cl⁻ secretion. Indeed, a loss of CFTR function, can also affect a number of other key ion channels, transporters and pumps which contribute to lung health by working together to ensure effective mucociliary clearance and innate immune defence mechanisms through the optimization of cell surface hydration, charge and pH [3]. In particular, the build-up of, and inability to clear, mucus in CF airways is due to an observed reduction in airway surface liquid (ASL) volume which is fundamentally a result of sodium hyperabsorption caused by dysregulation of the epithelial sodium channel (ENaC) in the cells lining the airways [4].

In this Review, we summarise the key areas covered in Symposium 6: *Cell Physiology and Ion Transport*, and highlight in particular recent developments in our understanding of CFTR structure and function as well as novel strategies to target ENaC. Other ion channels, such as the TMEM16A chloride channel and the calcium-activated potassium channel KCa3.1, and ion transporters are also presented as alternative pathways to restore surface hydration and pH in CF airways by increasing chloride and/or bicarbonate secretion. These approaches, summarized in **Figure 1**, offer very attractive targets for pharmacological intervention, and importantly could complement current drug therapies focused on the correction of CFTR mutations which together could result in the development of a broader arsenal of disease-modifying treatments for CF.

2. Recent developments in our understanding of CFTR structure and function

The CFTR chloride channel is a member of the family of ATP Binding Cassette (ABC) proteins, and is built from two homologous halves each containing a transmembrane domain (TMD) followed by a cytosolic nucleotide binding domain (NBD). In CFTR these two halves are linked by the unique cytosolic regulatory (R) domain [5] which inhibits channel activity unless phosphorylated by cyclic AMP-dependent protein kinase (PKA) [6, 7]. Unlike in other ABC proteins which are mostly active transporters, in CFTR the TMDs form a transmembrane anion-selective pore. Nevertheless, the molecular motions that drive uphill substrate translocation in ABC proteins but pore opening and closing (gating) in CFTR are highly conserved. The CFTR channel pore opens upon dimerization of its two NBDs, and closes upon disruption of this dimer following ATP hydrolysis [8]. In the tight NBD dimer canonical motifs

of both NBDs form two non-equivalent composite ATP binding sites. Composite site 2, formed by Walker motifs of NBD2 and the signature sequence motif of NBD1, is catalytically active, and hydrolyses ATP in each gating cycle. In contrast, composite site 1, formed by Walker motifs of NBD1 and the signature sequence motif of NBD2, is catalytically inactive, and keeps ATP bound throughout several gating cycles [9, 10]. Thermodynamic studies suggest that the pore opening conformational transition is initiated by tightening of the site-2 NBD interface, and that movements in this composite site are already completed in the opening transition state [11].

In contrast, little is known about the role and precise timing of molecular motions in composite site 1. Although profound effects on channel gating kinetics of site-1 perturbations suggest gating-associated motions also take place in this site [12, 13], the physical extent of such motions is debated [14-16]. Analysis of energetic profiles of channels gating in the absence and presence of ATP indicate that ATP bound at the dimer interface stabilizes the open state relative to the opening transition state, suggesting that this interface undergoes rearrangements between the transition state and the open state [17]. Insofar as motions at the site-2 interface are likely completed in the transition state, one possible explanation is that these further rearrangements occur at the site-1 interface. Recent thermodynamic studies presented at the Symposium indeed support such an interpretation, and suggest delayed movement in site 1 relative to site 2 during pore opening.

As described above, the major “driving force” for opening CFTR’s gate is attributed to ATP binding and subsequent NBD dimerization at composite site 2 [8, 18], but how ATP binding at composite site 1 contributes to this process is unclear. Although it has been shown that mutating the conserved Walker A lysine (K464) at this site decreases the apparent affinity of ATP for CFTR gating by more than 10-fold [18-20], on the contrary, reported that mutations such as K464A or W401G, which presumably weaken ATP binding at composite site 1, do not affect the sensitivity of CFTR to ATP. These two latter reports, however, show a shortening of the open time by these mutations. If closing of CFTR’s gate is driven by disruption of the NBD dimer, normally controlled by ATP hydrolysis at composite site 2 [21], this result suggests that the structure/function status of site 1 may affect the hydrolysis rate at composite site 2, and/or the stability of the pre-hydrolytic NBD dimer.

A potential role of composite site 1 in CFTR gating was revealed by a study that used the high-affinity ATP analog N⁶-phenylethyl ATP (P-ATP) as an alternative ligand [16]. This study led to a proposition that a complete gating cycle is coupled to ATP hydrolysis at composite site 2 while composite site 1 remains occupied. The data presented at the Symposium extend this idea and suggest that gating becomes much less effective when composite site 1 is empty, probably because an unoccupied site 1 enables a wider separation of the two NBDs and hence hinders NBD dimerization at composite site 2. Indeed, a pathogenic mutation G1349D that presumably prevents association of NBDs at composite site 1 drastically dampens ATP-dependent gating [22]. As both composite ATP-binding sites are located at the NBD interface, it seems not surprising that a functional interaction between them should take place. Deciphering the precise nature of this interaction awaits further studies.

This continued expansion of our knowledge of CFTR structure and function will undoubtedly contribute to ongoing drug development in the field, recent advances in which have been extensively reviewed elsewhere [2, 23].

3. Targeting ENaC

Within the airways, the epithelial sodium channel (ENaC) has been found to be solely responsible for the absorption of Na⁺ and, in CF, its dysregulation is now known to directly contribute to mucus stasis and impaired mucus clearance [24].

ENaC is composed of three structurally related subunits (α , β and γ), which include two membrane-spanning domains connected by a large extracellular loop [25, 26]. Although ENaC can be regulated by multiple pathways e.g. cAMP [24] and SPLUNC-1 [26], it is activated by the proteolytic processing of its subunits leading to an increase in channel conductance [24, 27]. The importance of proteases in ENaC activation in CF is further supported by evidence indicating that wild-type CFTR physically associates with ENaC, impedes proteolysis and suppresses channel opening, whereas Δ F508 CFTR fails to protect ENaC from proteolytic cleavage and stimulation [28].

Channel activating proteases (CAPs) predominantly belong to the trypsin-like family of serine proteases as studies investigating ENaC processing and activation have, to date, determined multiple Arg and Lys cleavage sites on α and γ subunits: e.g., γ Lys¹⁹⁴ (plasmin) [29]; α Arg²⁰⁵, α Arg²³¹ and γ Arg¹⁴³ (furin); γ Lys¹⁸⁶ (prostasin; CAP-1) [30] and γ Arg¹³⁸ (CAP-2) [31]. Neutrophil elastase, which is associated widely with chronic airways disease, can also cleave ENaC γ although a pre-processing step by furin is thought to be required for complete elastase-induced activation of ENaC [27]. Although, the specific CAPs responsible for ENaC activation in CF airways have yet to be defined, both host and bacterial enzymes are implicated, the impact of their activities further exacerbated by the protease-antiprotease imbalance associated with disease progression. A broad spectrum approach to the inhibition of trypsin-like serine proteases in CF has however, been validated using both macromolecular protease inhibitors (aprotinin) and the low molecular weight inhibitor Camostat, which were found to attenuate ENaC and improve mucociliary clearance [32, 33].

Recent work, presented at the Symposium, describes the development of a novel rationally-designed compound (QUB-TL1) whose inhibition of excessive apical CAP activity is restricted to the extracellular surface of airway epithelial cells [34]. The broad spectrum inhibition of putative CAPs, to include human airways trypsin-like protease (HAT), prostasin, matriptase and furin resulted in diminished ENaC-mediated Na⁺ absorption in CF primary human airway epithelial cells (hAECs) and the internalization of a prominent pool of cleaved (active) ENaC γ from the cell surface. Furthermore, diminished amiloride-sensitive ENaC activity correlated with an increase in ASL height and restored normal mucociliary clearance. A further novel trypsin-like inhibitor, NAP-858 was also reported for the first time. QUB-TL1 and NAP-858 dampen CAPs-ENaC signalling which improves hydration status and mucociliary clearance in CF airway epithelial cell cultures and may provide a mechanism to delay or prevent the development of CF lung disease in a manner independent of CF transmembrane conductance regulator mutation.

A number of other approaches targeting ENaC function as a treatment for CF are at various stages of development [35]. The in vitro and in vivo efficacy of SPX-101, peptide mimetic of SPLUNC1 has recently been reported [36]. SPX-101 has been shown to bind to ENaC and to promote internalization of α , β and γ subunits in both CF and healthy primary hAECs, which similarly to QUB-TL1 caused a significant decrease in amiloride-sensitive current. In vivo studies found that once daily dosing with SPX-101 had the ability to increase survival of β ENaC

transgenic mice to >90% and increased mucus transport in both the β ENaC mouse and sheep models of CF.

Genomic approaches to ENaC inhibition has also been extensively considered and to date has involved the design of siRNA to α ENaC and delivery to airway cells using nanoparticle formulations [37, 38]. Delivery of siRNA using both liquid nanoparticle formulations [37] and a novel self-assembly nanocomplex formulation [38] were able to silence expression of the α ENaC subunit gene and warrant further evaluation as potential novel inhaled therapeutics for CF.

4. Alternative ion channels and transporters in CF

Beyond CFTR and ENaC, other ion channels and transporters are being investigated as potential alternative pathways to restore airway surface hydration (Figure 1A) and pH (Figure 1B) by increasing chloride and/or bicarbonate secretion.

Airway epithelia respond to Ca^{2+} agonists, such as ATP and UTP, by a large increase in Cl^- secretion, and therefore possess a Ca^{2+} -activated Cl^- conductance. In 2008, 3 different research groups have identified TMEM16A, also named Anoctamin-1, as a calcium dependent chloride channel expressed in airway epithelial cells [39-41]. Moreover, it has been shown that, besides Cl^- , this channel is permeable to HCO_3^- [42]. Theoretically, activating this channel could therefore increase ASL hydration and pH. However, it has been reported that TMEM16A expression is increased in response to pro-inflammatory stimuli, associated with goblet cell metaplasia [43] and increased in airways of asthmatic patients [44]. Nevertheless, small molecules activating or inhibiting this channel are being developed as its role in CF pathophysiology is further investigated [45, 46].

The SLC26 family encodes anion exchangers and channels, two of which are of particular interest in the search for alternative pathways in CF lung pathophysiology. SLC26A4, also known as pendrin, is an electroneutral $\text{Cl}^-/\text{HCO}_3^-$ exchanger expressed in epithelial cells of many organs, including the airways, and plays an important role in the lung innate immune defence by transporting thiocyanate [47]. In Calu-3 cells, it is mainly responsible for HCO_3^- secretion [48] and could therefore be targeted in order to increase ASL pH in CF. However, a recent study reported that pendrin inhibition increased ASL hydration [49]. Thus establishing the therapeutic potential of the modulation of pendrin activity may prove to be difficult. SLC26A9, unlike the other members of the SLC26 family, is a Cl^- channel involved in resting and cAMP-regulated Cl^- secretion. Multiple reports provide strong evidence for this channel as a modifier gene in CF and other lung diseases [50] and Single Nucleotide Polymorphisms in this gene have been associated with severity of lung pathology in individuals with CF and asthma. However, regulation of this channel has yet to be fully understood and to date, no specific modulator of its activity has been identified. Therefore, although SLC26A9 is a very strong candidate for an alternative Cl^- pathway in CF, much progress is still required before it can be fully considered as a therapeutic target.

Basolateral K^+ channels maintain the membrane potential and provide the driving force for Cl^- secretion. Moreover, it was shown that activating a Ca^{2+} regulated K^+ channel (KCNN4) with 1-EBIO increased Na^+ absorption across CF airways epithelial cells [51]. Therefore, it appears that modulating K^+ channel activity could modulate ASL hydration and might restore an efficient mucociliary clearance in CF cells.

Finally, recent studies have demonstrated the importance of ASL pH in airway hydration, bacterial killing, antimicrobial peptide activity and mucus rheology. Thus adjusting H^+ and HCO_3^- secretion could, theoretically, improve the CF lung physiology. Modulating Na^+/H^+ exchangers, H^+ or HCO_3^- conducting channels and transporters could also increase ASL pH, reversing different hallmarks of the CF lungs, regardless of the CFTR mutation. Several reports showed the prominent role of ATP12A, an H^+/K^+ -ATPase, in the acidic ASL found in CF airways [52, 53] and inhibiting this pump could therefore be beneficial for CF airways.

5. Conclusion

It is clear that cell physiology and ion transport in CF is complex and requires an understanding not just of the disease-causing CFTR gene, and the structure and function of the CFTR protein but of the number of other ion channels and transporters that contribute to the electrophysiological balance within the airways, many of which are impacted by the loss of functional CFTR. When the CFTR gene and its association to CF was discovered in 1989 by Dr Lap-Chee Tsui and colleagues it was not expected that gene therapy and ultimate cure for CF would remain a holy grail [5]. Increases in life expectancy over the last number of decades have instead been due to improvements in disease management and treatment [1]. It is however hoped that recent progress in the development of CFTR modulators (potentiators, correctors, amplifiers, read-through agents and stabilisers) in various combinations will provide further opportunity to improve both quality of life and the life expectancy of those living with CF, although it will not be without its challenges [23].

An alternative, potentially complementary and highly attractive strategy is the targeting of other ion channels which offers an opportunity to develop ion transport modulation therapies irrespective of an individual's CFTR genotype [2, 50]. A number of pharmacological and genomic approaches to inhibit ENaC are at various stages of development [34, 36-38]. The identification of alternative chloride channels and potassium channels involved in the maintenance of ion balance and pH in the airways also offer new targets for drug development [39, 49]. A multi-track approach to enable Cl^- secretion by a reconstitution of the defective CFTR or through the activation of alternative Cl^- channels and by blocking ENaC to prevent Na^+ hyperabsorption will therefore be critical to ensure future improvements in the health of individuals with CF.

Conflicts of interest:

The authors declare there is no conflict of interests between them and regarding the publication of this paper.

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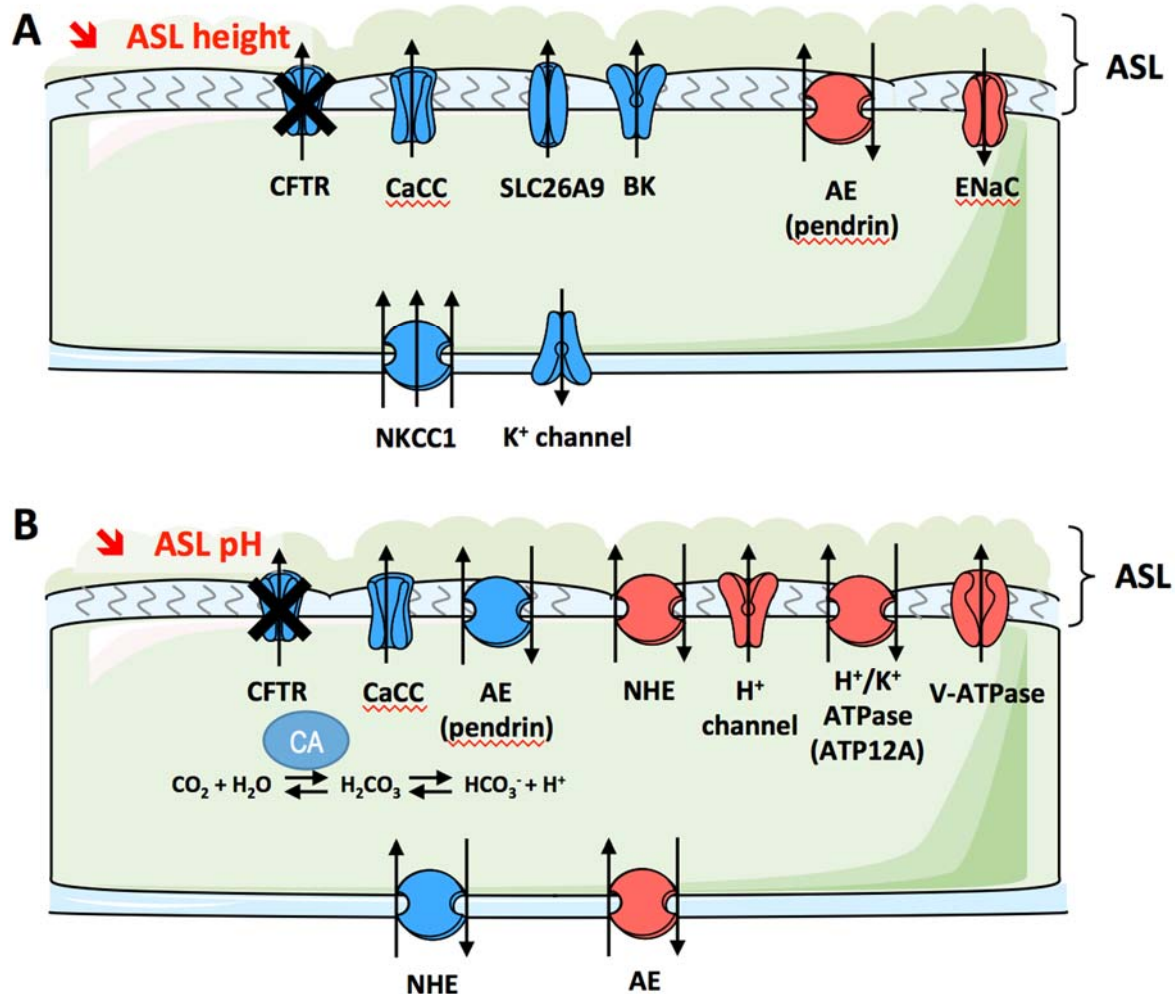


Figure 1. Alternative channels and transporters for the regulation of ASL height (A) and ASL pH (B), in CF.

A. Modulation of Cl⁻ and Na⁺ transport involves increasing anion and fluid secretion (by activating the blue channels and transporters) and/or decreasing Na⁺ and fluid absorption (by inhibiting the red channels and transporters). Anion secretion can be increased by activating Ca²⁺-activated Cl⁻ channels (CaCC), such as TMEM16A, or Cl⁻ channels, such as SLC26A9 on the apical membrane. K⁺ secretion on the apical surface can also regulate ASL volume. On the basolateral membrane, a Na-K-Cl cotransporter (NKCC1) is the limiting factor for Cl⁻ entry into the cells and K⁺ recycling through basolateral K⁺ channels, such as KCNQ1, provides the driving force for transcellular Cl⁻ secretion. Inhibition of ENaC reduces Na⁺ hyperabsorption and fluid absorption which increases ASL volume. Inhibition of pendrin, an anion exchanger (AE) has also been shown to increase airways hydration

B. Modulation of HCO₃⁻ and H⁺ transport involves increasing apical HCO₃⁻ secretion or basolateral H⁺ secretion (through blue channels and transporters) and/or decreasing apical H⁺ secretion and basolateral bicarbonate (HCO₃⁻) secretion (through red channels and transporters). Theoretically, activation of any apical HCO₃⁻ transporter could increase ASL pH, such as CaCC or pendrin. Inhibiting apical Na⁺/H⁺ exchangers (NHE; such as NHE3/SLC9A3, a modifier gene associated with severity of CF lung disease), H⁺ channels, H⁺/K⁺-ATPase or V-ATPase could also increase ASL pH. In the cytoplasm, the carbonic anhydrase (CA) is involved in the CO₂/HCO₃⁻ buffering system and could contribute to the regulation of ASL pH by increasing intracellular HCO₃⁻ concentration. In the basolateral membrane, activating NHE could prevent apical H⁺ secretion and inhibiting anion exchange could sustain intracellular HCO₃⁻ concentration required for its apical secretion.